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Influence of exogenous phytohormones on the functional activity of apical meristematic cells in wheat seedlings.

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The functioning of plant apical meristems is controlled by the hormonal regulatory system, which operates at all stages of plant ontogenesis. A topical problem is the identification and further study of molecular markers involved in the perception of a hormonal signal and its transmission to the plant cell genome.

Our preliminary work has found that the meristematic cells of the wheat apex are characterized by the presence of a marker protein called the proliferative antigen of initials (PAI), whose content in root and stem meristematic cells correlates with their mitotic index (i.e., it defines the extent of activity of these cells (Evseeva et al. 2009). The suggestion has been made that PAI is associated with the perception of an auxin or cytokinin signal and its transmission to the cell genome. Our aim was to examine the influenceof exogenous auxins and cytokinins on the functional activity of meristematic cells in the seedllings of wheat cultivar Saratovskaya 29.

The root system of 5-day-old seedlings was treated with solutions of indole-3-acetic acid (IAA; 1 and 0.1 mg/L) and 6-benzilaminopurine (6 BAP; 1 and 0.1 mg/L). The activity of meristematic cells was assessed by the results of determination of the cells mitotic index and by comparative immunochemical estimates of PAI content in these cells.

IAA at 1.0 and 0.1 mg/L enhanced the mitotic activity of the root meristematic cells 2- and 2.5-fold, respectively. The PAI content of the apical meristems changed insignificantly. In turn, in response to 6-BAP at 0.1 mg/L, cellular mitotic activity increased 2-fold and PAI content increased 1.2-fold. These results suggest that PAI involved in the perception of signals from gormones of the cytokinin series and their transmission to the plant cell genome.

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The programmed cell death in winter wheat suspension culture at low temperatures.

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Programmed cell death (PCD) is the genetically controlled process of the organized destruction of superfluous or defective cells (Krishnamurthy et al. 2000; Kingston-Smith et al. 2008). The mechanisms of PCD are well known in animals, whereas many features of this process in plants are needed to investigate. PCD in plants plays a crucial role in real-

izing of development program, response to pathogens and different abiotic stress (Heath 1998; Jones 2001; Gao et al. 2008). PCD in plant cells is accompanied by a number morphological and biochemical changes, just as in animal cells, and include chromatin condensation with subsequent nuclei disintegration and DNA fragmentation, concentration and vacuolization of the cytoplasm, protoplast condensation, release of cytochrome c from the mitochondria, activation of endonucleases and caspase-like proteins, generation of reactive oxygen species, and dependence of the death process on ATP level in the cell and protein synthesis de novo (Reape et al. 2008).

The available literature data about possibility of induction and development PCD process under cold conditions are not numerous (Koukalová et al. 1997; Ning et al. 2002). In these works, the possibility of PCD activation is investigated under low temperature treatment. Nothing is known about opportunity of subzero temperatures to cause PCD in plants. The aim of our work was to investigate conditions for PCD activation in a winter wheat suspension culture during treatment with low and subzero temperatures.

Materials and methods. Suspension-cultured cells of *T. aestivum* were grown in the dark at 26°C under continuous shaking in Murashige and Scoog (MS) medium containing sucrose (3%), thiamine (1.0 mg/L), pyridoxine (0.5 mg/L), nicotinic acid (0.5 mg/L), 2.4-D (2.5 mg/L), inositol (0.01%), and sodium dithiocarbamate (0.0005%). Suspensions were subcultured every 14 days using 2:7 dilutions. All treatments were carried out using log-phase cells 8 days after subculture. Suspension-cultured cells were subjected to cold hardening for 7 days at 8°C or 4°C and following short-term treatment (–8°C, 6 hours). After these treatments, suspension cells were moved under the control conditions (26°C) for 3, 6, and 10 days. Evans' blue staining of cell culture was used to determine the number of dead cells and cells with condensed protoplasts (Baker and Mock 1994). At least three independent experiments were performed with more than 500 cells counted per conditions. The quantity of stained cells and cells with condensed protoplasts were calculated using light microscope Axiostar plus (Carl Zeiss, Germany). Images were made using inverted fluorescent microscope AxioObserver Z1 (Carl Zeiss, Germany) with digital monochrome camera AxioCam MRm3 and the AxioVision Rel.4.7.2 software.

Results and discussion. Our experiments showed that reaction of suspension cultures hardened at different temperatures to transferring in the control conditions and following treatment with subzero temperatures differ greatly. About 15% of the cells were dying during culture treatment with 8°C, but during the following 10 days of the experiment at 26°C, cell death stopped (Fig. 1). Furthermore, the subsequent cold shock (the treatment with subzero temperature, CS) did not cause the mass mortality of suspension cells and during our experiment. The decrease in the quantity of living cells in the culture exposed to preliminary hardening at 8°C and then CS was only about 10–15% compared to the control culture (Fig. 1). The control culture treatment with subzero temperature caused the death of 15% of cells during exposure and 45% after transferring the culture in the control conditions (Fig. 1). The defense mechanisms became apparent in the suspension culture exposed preliminary cold hardening at 8°C. They allowed cells to withstand CS.

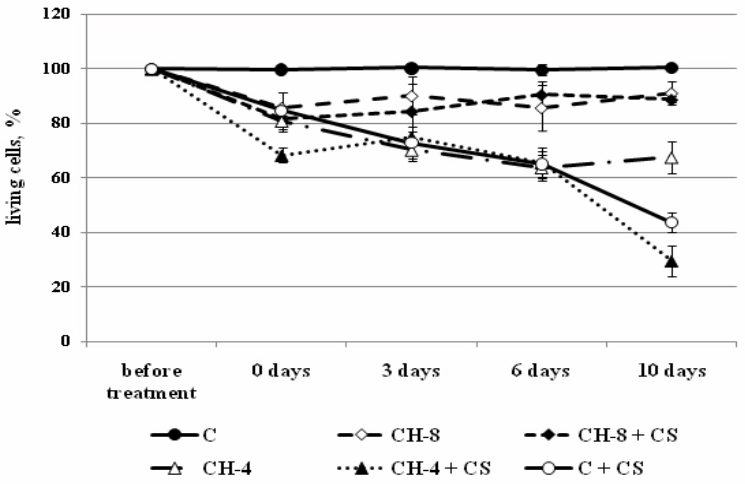

Fig. 1. The influence of subzero temperature (–8ºC, 6 h) on the dynamics of the cell death in the control and hardened suspension culture of winter wheat. C – control suspension culture without any treatment; CH-8 – cold hardening at 8ºC, 7 days; CH-4 – cold hardening at 4ºC, 7 days; CS – cold shock (treatment with subzero temperature) (M ± m; n=3–6).

Other tendency was observed in experiments at 4°C. The quantity of dead cells during this treatment was slightly greater than that in the culture hardened at 8°C (about 5%), but after transferring this suspension culture to the control conditions, the process of cell death continued and 70% of cells dyed during following 10 days of the experiment (Fig. 1). This quantity was greater than that of the respective percent of the dead cells in the control culture after the treatment with subzero temperature. In this connection, it is possible that metabolic state of the cells determined their

further existence (death program or adaptation). The process of PCD on the first stage is reversible, therefore after transferring the culture to the control conditions, those cells in which development of PCD has passed 'the point of no return' were dying during the 6 days of the experiment. At the same time, those cells in which the adaptation mechanisms have been formed or the development of PCD was at the reversible initial stages, returned gradually to the normal vital functions (O'Brien et al. 1998). The process of cell death caused the treatments with low and subzero temperatures to a have gradual, prolonged character; the process developed during several days and not at the same time as the CS treatment, but after it (Fig. 1). This fact allowed us to suppose the active character of the death in suspension culture. Thus, one of the important features characterizing active, genetically programmed cell death became apparent, the development of the process takes a long time. Reape et al. (2008) observed that PCD in plants is slower than in animals and develops during several hours, rarely during one day. In our experiments, PCD was connected with features of stress to low and subzero temperatures.

PCD in plants and animals depends on activity of many enzymes and protein and ATP synthesis (Williams and Dickman 2008). At subzero temperatures or the temperatures near 0°C, many enzymes in the cell denaturate because of a decrease in hydrophobic pressure providing their functional activity, in particular disintegration of the ATP-synthase complex (Finkelstein and Ptitsyn 2002). During cold denaturation of protein, the forming of 'boiling up' of a protein rather than 'molten globule' is observed is significant. Thus, the recovery of disturbed bonds in a spatial pattern takes much time after cold treatment and explains the slow character of the death process in our experiment. Koukalová et al. (1997) have shown the development of PCD under low temperature treatment of tobacco cell culture during 5 weeks.

The shrinkage of the cell and the condensation of the protoplast away from the cell wall is the one of more prominent features of PCD (Reape et al. 2008) and such changes are easy to observe in a light microscope (Fig. 2). The quantity of the cells with condensed protoplasts in the suspension culture hardened at 8°C was equal to that of the control level, whereas after the treatment with 4°C, cell death is accompanied by a 15–18% increase (Fig. 2). Subsequent treatment with subzero temperatures led to protoplast condensation both in the control culture and in the culture preliminary hardened at 4°C (Fig. 2), agreeing with the data of the cell death process after the treatment (Fig. 1, p. 266).

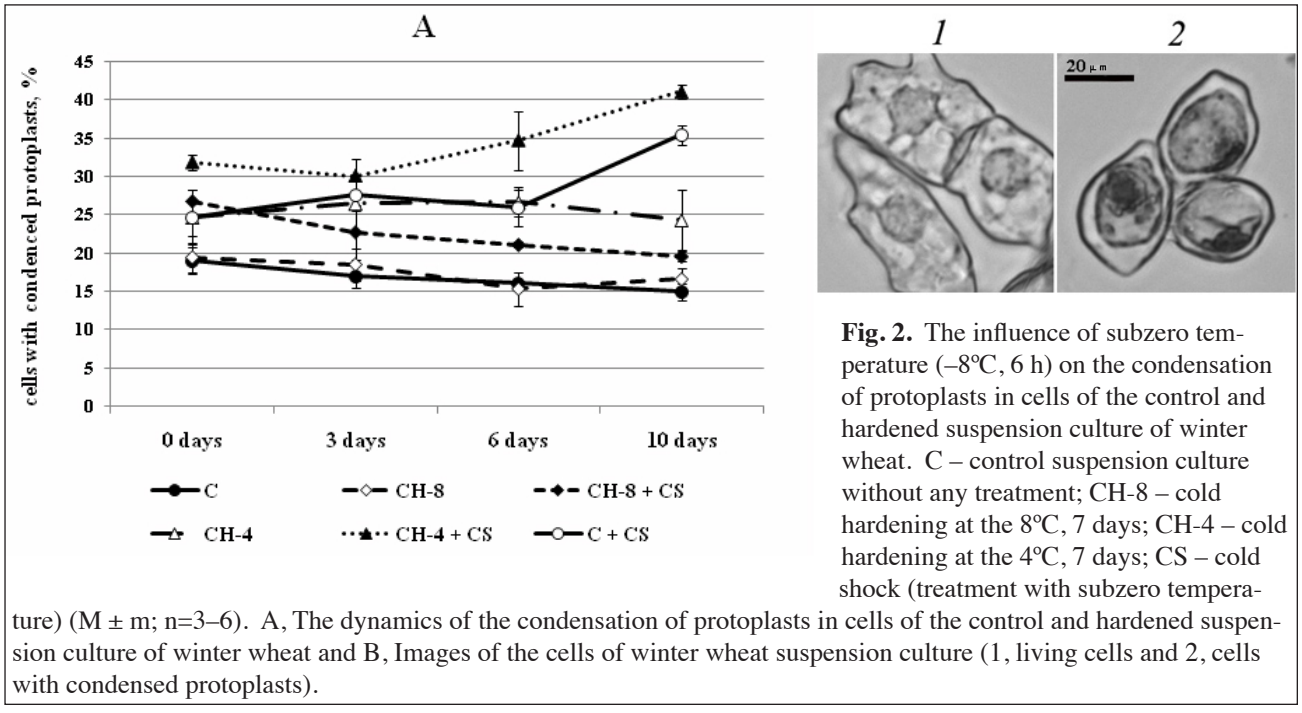

Fig. 2. The influence of subzero temperature (–8ºC, 6 h) on the condensation of protoplasts in cells of the control and hardened suspension culture of winter wheat. C – control suspension culture without any treatment; CH-8 – cold hardening at the 8ºC, 7 days; CH-4 – cold hardening at the 4ºC, 7 days; CS – cold shock (treatment with subzero temperature) (M ± m; n=3–6). A, The dynamics of the condensation of protoplasts in cells of the control and hardened suspension culture of winter wheat and B, Images of the cells of winter wheat suspension culture (1, living cells and 2, cells with condensed protoplasts).

These results allow us to conclude that low temperature may be both necessary for forming of mechanisms of low-temperature adaptation and a factor for PCD activation in suspension culture of winter wheat. At the same time, PCD under low temperature conditions is a slow process, which is accompanied by respective morphological changes.

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The antioxidant function of alternative oxidase and uncoupling proteins in winter wheat mitochondria under cold hardening.

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Cold hardening under low nonfreezing temperatures (first phase of hardening) attacks the ability winter crops to tolerate unfavorable freezing temperature. The acquisition of additional freezing tolerance is the second phase of hardening and takes place when plants are exposed under subzero temperatures (–2–3°C). Cold hardening of winter wheat is known tocause the activation of alternative oxidase (AOX) (one of terminal oxidases of mitochondrial electron transport chain, ETC) (Grabelnych et al. 2003, 2004; Sugie et al. 2006; Mizuno et al. 2008). The ability of alternative pathway (AP) relating to AOX functioning to respond to low-temperature conditions is one of the genetic factors determining cold/frost resistance in winter wheat (Sugie et al. 2006; Mizuno et al. 2008). One function of AOX in plant cells is the decrease of reactive oxygen species (ROS) formation (Popov et al. 1997; Maxwell et al. 1999; Moller 2001) that can be first line of mitochondria protection from oxidative stress (Moller and Kristensen 2004). The uncoupling proteins can carry out similar function in plant mitochondria (Kowaltowski et al. 1999; Considine et al. 2003). But, in contrast to AOX, uncoupling proteins are able to operate under increased ROS content (Rhoads et al. 2006). Sluse et al. (1998) have shown that an increase of free fatty acids (FFA) concentration blocked AOX activity causing activation of uncoupling proteins in vitro. We suppose that the in vivo increase of FFA content in mitochondria along with increase of ROS can regulate AOX and uncoupling proteins activities under stress (particularly, induced by low and subzero temperatures). Our aim was to study of AOX and uncoupling proteins activities under cold hardening in winter wheat seedlings and to detect theirs antioxidant function.

Materials and methods. Three-day-old etiolated seedlings of cold-resistant winter wheat cultivar Irkutskaya ozimaya were germinated on moist paper at 26°C and used as a control. For cold hardening, 2.5-day-old etiolated seedlings germinated at 26°C at 2–3°C for 7 days (first phase) and then placed in an incubator at –2°C for 2 days (second phase). The efficiency of cold hardening was estimated by synthesis of dehydrins. Mitochondria were extracted from shoots by differential centrifugation and purified on Percoll gradient (Pobezhimova et al. 2001). The isolated mitochondria were resuspended in the medium contained 40 mM MOPS-KOH buffer (pH 7.4), 300 mM sucrose, 10 mM KCl, 5 mM EDTA and 1 mM $MgCl_2$. The concentration of mitochondrial protein was analysed by Lowry. Integrity of mitochondrial outer membrane from was calculated on rate of ascorbate-dependent cytochrome-c-induced KCN-sensitive oxygen consumption in presence and absence of 0.04% Triton X-100 and was 92-93%. Mitochondrial activity was recorded polarograph-

ically at 26°C using a closed-type platinum electrode in a 1.4-ml cell. The reaction medium for AOX determination contained 300 mM sucrose, 20 mM MOPS-KOH buffer (pH 7.4), 5 mM $MgCl_2$, 10 mM EDTA, 0.1% bovine serum albumin (BSA) clear free fatty acids, 8 mM succinate (Suc), 5 mM glutamate (Glu), 3 mkM rotenone (Rot), 200 mkM ATP, 1 mM pyruvate, and 5 mM ditiotreitol. The concentrations of inhibitors of respiratory chain were: Rot (3 mkM), antimycin A (A-A) (20 mkM), benzhydroxamic acid (BHAM) (1 mM) and KCN (0.4 mM). The reaction medium for PUMP determination contained 150 mM sucrose, 10 mM Tris-HCl (pH 7.4), 65 mM NaCl, 5 mM EDTA, 0.33 mM EGTA, 8 mM Suc, 5 mM Glu, 3 mkM Rot, 200 mkM ATP, 1 mM BHAM, and 8 mkM linoleic acid (LA). ROS content in isolated mitochondria evaluated by 1 mkM H2DCF-DA (2',7'-dichlorofluorescein diacetate). Fluorescence of DCF was measured by using spectrofluorophotometer SHIMADZU RF-5301PC (Japan) with excitation and emission wavelengths set at 480 nm and 524 nm, respectively. All the experiments were performed on 3–6 separate mitochondrial preparations, arithmetic means and standard error are presented.

Results and discussion. First phase of cold hardening was accompanied by 32-41% decrease of state-3 respiration in winter wheat mitochondria whereas two phases of cold hardening lead to a 65–66% decrease in state-3 respiration. The decrease in the mitochondrial cytochrome pathway (CP) from 77% (control seedlings) to 53% and generation of ROS by mitochondria (a 1.5-fold increase in comparison with control) occurred during first phase of cold hardening (Fig. 3). At the same time, an approximately 1.8-fold activation of AP occured (with 22% to 40%) that was accompanied by synthesis of AOX stress isoforms. Still more ROS generation by mitochondria (2.8-fold) was observed under second phase of cold hardening) (Fig. 3) and at the same time the inhibition of AP (to 17%) and the increase of CP (to 73%) were observed. We suppose that the increase in ROS generation by winter wheat mitochondria under cold hardening is related to signal function of these molecules. AOX is protein of nuclear encoding, transmission of signal from mitochondria into nucleus and induction of nuclear genes consequently of mitochondrial signal pathway realization possible to allow plants to support cell homeostasis in changing environment (Rhoads et al. 2006). Activity of AOX may be able to estimate power of this signal pathway (Vanlerberghe et al. 2009).

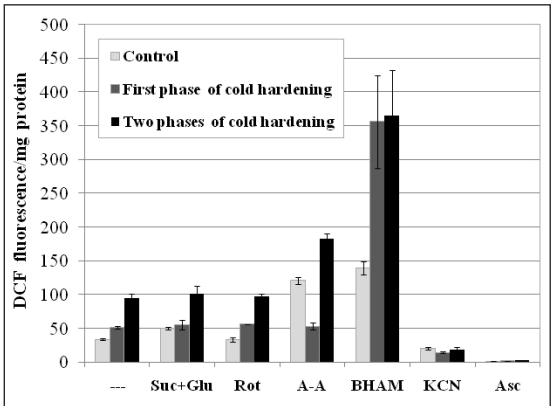

Fig. 3. The ROS content in mitochondria isolated from control and cold hardened seedlings of winter wheat (M±S.E; n=8–10). Control, before cold hardening; first phase of cold hardening, 2–3°C, 7 days; second phase of cold hardening, 2–3°C, 7 days + –2°C, 2 days; ---, without any additions; Suc + Glu, with 8 mM succinate and 5 mM glutamate added; Rot, 3 mkM rotenone added; A-A, 20 mkM antimycin A added; BHAM, 1 mM benzhydroxamic acid added; KCN, 0.4 мM KCN added; and Asc, 60 mM ascorbic acid added.

Succinate and respiratory inhibitors A-A and BHAM increased generation of ROS (1.5-, 3.6-, and 4.1-fold) in mitochondria from control seedlings while KCN inhibited generation of ROS by mitochondria (about 41%) (Fig. 3). These data agree with literature data about ability of A-A and hydroxamic acids to cause an increase of ROS generation by plant mitochondria (Popov et al. 1997). At the same, time suc and A-A did not cause an increase of ROS generation, but BHAM caused a 7-fold the increase in ROS generation in mitochondria from seedlings after first phase of cold hardening (Fig. 3). Taking into consideration that on this stage of cold hardening activation of AP occurs, we may conclude that antioxidant function of AOX is one of cause of the decrease of substrate- and A-A-dependent ROS generation in winter wheat mitochondria and greater ROS generation under addition of BHAM also supports this fact. The second phase of cold hardening also was accompanied the decrease of succinate-dependent ROS generation by mitochondria and lesser ability of A-A to generate ROS in comparison with control mitochondria (a 2-fold), but the effect of BHAM was similar to the control mitochondria (Fig. 3). These data show that antioxidant function of AOX during second phase of cold hardening is carried out in lesser degree than during first. Incubation of winter wheat mitochondria with ascorbic acid leads to full neutralization of ROS and Rot did not influence on ROS production of mitochondria (Fig. 3).

The accumulation of hydrogen peroxide in plant mitochondria during oxidation of suc and BHAM and A-A addition is related to significant increase of superoxide radical anions production (Popov et al. 1997). Superoxide radical anion is known to be an unstable compound and rapidly neutralized to hydrogen peroxide with the participation of superoxide dismutase. Hydrogen peroxide is more stable compound and can diffuse in cell on significant distance that may determine its ability to be a signal molecule.

The decrease of succinate-dependent ROS generation by mitochondria and lesser ability of A-A to produce of ROS during second phase of cold hardening (Fig. 3) may indicate on function of uncoupling proteins. We carried out analysis of state-4 respiration rate, respiration control by Chance-Williams (RC) and ADP/O ratio in winter wheat mitochondria from seedlings subjected to cold hardening. The rate of state-4 respiration in absence of LA was remained constant but the decrease of state-3 respiration rate occurred that was accompanied by the decrease of RC and ADP/O ratio in mitochondria from hardening seedlings. The decrease of ADP/O ratio was maximal under two phases of cold hardening (about 80%). We found that the addition of LA to mitochondria leads to stimulation of state-4 respiration: about 34%, 15%, and 47% in the mitochondria from control, hardened under low, and subzero temperatures seedlings, respectively. The decrease of RC and ADP/O ratio also was most expressed after two phases of cold hardening. The absence of significant stimulation of state-4 respiration in mitochondria after first phase of cold hardening possibly is explained by increase in these conditions of AOX activity. We estimated ROS in the mitochondria of control and hardened seedlings in presence of uncoupling proteins activators and inhibitors. Preliminary results show that activation of uncoupling proteins under incubation of mitochondria with LA effective decreases ROS generation by mitochondria whereas GTP (inhibitor of uncoupling proteins) vice-versa increases ROS.

Thus, first phase of cold hardening leads to inhibition of CP in winter wheat mitochondria, the increase of their ROS content and switch of electrons transport from CP to AP. Under this, likely, ROS carries out the function of signal molecules regulating expression and synthesis of AOX and activation of AP. Antioxidant function of AOX during first phase of cold hardening may present significant component of low-temperature adaptation of winter crops. Under more significant increase of level ROS in mitochondria (not damaging subzero temperatures) uncoupling proteins can replace AOX.

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ITEMS FROM UKRAINE

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New morphological trait in the genus Triticum L.

O.V. Tverdokhleb.

In awned forms of the genus *Triticum*, awns usually are jagged in varying degrees. Smooth awns are relatively rare, they are found only in the cultivated tetraploid wheats *T. turgidum* subsps. *durum* and *turgidum* (Dorofeev 1972) and forms with pubescent awns still have not described in wheat (Tsvelev 1976; Dorofeev 1979). We found such forms in the progeny from a cross '*T. timopheevii* subsp. *timopheevii* / *T. turdigum* subsp. *durum* cultivar Spadshchyna (Fig. 1). In these forms, awn pubescence is a continuation of the pubescence from the top of the lemma and extends to a length of about 2.5 cm, regardless of awn length. In hybrid F_1 plants, the awns were jagged but not pubescent.

Fig. 1. Awn pubscence in the progeny from a cross '*T. timopheevii* subsp. *timopheevii* / *T. turdigum* subsp. *durum* cultivar Spadshchyna. Awn pubescence is a continuation of the pubescence from the top of the lemma and extends to a length of about 2.5 cm, regardless of awn length.

Of the 154 florets of hybrid F_1 plants pollinated with Spadshchyna, 22 seeds were obtained and 12 F_1BC_1 plants were grown. From 12 spikes in the F_2BC_1, four were fertile with seed set from 2.8 to 47.4%. All the spikes had light glumes with light awns and were slightly pubescent. When 25 seeds were sown, plants of F_3BC_1 were obtained, which were divided into five groups.

1. Spikes of dark coffee color, awned, glumes not pubescent, awns dark and pubescent. These five plants were derived from spontaneous pollination of the hybrid by pollen of *T. persicum*. Their fertility was close to zero; only one shriveled seed was found.
2. Spikes light with black pubescent awns, glumes not pubescent. This group included two plants with spike fertility of 18.2–25.0%.
3. Spikes light with black pubescent awns, glumes pubescent. We have assigned to this group five plants with a fertility of 19.2–34.2%.
4. Spikes with light pubescent glumes and awns. To this group were assigned eight plants with fertility from 2.6–3.1%.
5. Spikes light, no pubescence, glumes not pubescent. The group included five plants with a fertility from 2.6–9.4%.